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The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens

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Abstract

H5N1 avian influenza viruses are continuing to spread in waterfowl in Eurasia and to threaten the health of avian and mammalian species. The possibility that highly pathogenic (HP) H5N1 avian influenza is now endemic in both domestic and migratory birds in Eurasia makes it unlikely that culling alone will control H5N1 influenza. Because ducks are not uniformly killed by HP H5N1 viruses, they are considered a major contributor to virus spread. Here, we describe a reverse genetics-derived high-growth H5N3 strain containing the modified H5 of A/chicken/Vietnam/C58/04, the N3 of A/duck/Germany/1215/73, and the internal genes of A/PR/8/34. One or two doses of inactivated oil emulsion vaccine containing 0.015 to 1.2 μg of HA protein provide highly efficacious protection against lethal H5N1 challenge in ducks; only the two dose regimen has so far been tested in chickens with high protective efficacy.

Keywords: H5N1; Influenza; Oil-emulsion; Vaccines; Efficacy; Ducks; Chickens

Introduction

The H5N1 avian influenza virus that emerged in 1996 in southern China (Xu et al., 1999) is now endemic in domestic poultry in the region and has continued to spread across Asia (Horimoto and Kawaoka, 2005; Lipatov et al., 2004). Descendants of this virus continue to ravage the poultry industries of Asia. The virus has infected 121 humans in Vietnam, Thailand, Cambodia, and Indonesia, killing 62. In the summer of 2005, this H5N1 virus caused a lethal outbreak of influenza in wild birds (mainly bar-headed geese) in Qinghai Lake in western China (Chen et al., 2005; Liu et al., 2005) and subsequently spread to poultry in Russia, Kazakhstan, Mongolia, and Turkey. The available evidence shows that the Z genotype of highly pathogenic H5N1 influenza virus is now endemic in wild migratory birds and will continue to spread across Europe.

Although quarantine, restricted movement, and culling of infected and adjacent flocks is the traditional method of controlling and eradicating highly pathogenic avian influenza viruses (Lee et al., 2005), other strategies, including improved biosecurity and vaccination, have been adopted in some countries. Hong Kong SAR, China successfully controlled H5N1 in 2004, although poultry in the surrounding countries were ravaged by highly pathogenic H5N1 avian influenza (Sims et al., 2003; Ellis et al., 2004). The changes adopted in Hong Kong were: (1) a ban on ducks and geese (the primary source of influenza) and quail (potential amplifiers) in the live poultry markets; (2) improved sanitation of live markets with two clean days per month; (3) vaccination of all chickens and serologic monitoring of sentinel unvaccinated chickens in each poultry shed to ensure absence of virus shedding. No H5N1 virus has been detected in domestic poultry or people in Hong

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Kong in 2004 or 2005; therefore, these strategies may successfully control the spread and interspecies transmission of H5N1 virus.

Vaccination of chickens and turkeys with inactivated, oil emulsion influenza vaccines is known to prevent influenza disease signs and reduce virus spread and shedding (Karunakaran et al., 1987; Stone, 1987; Brugh et al., 1979). A second strategy for vaccinating chickens is infection with recombinant fowlpox virus containing an influenza virus HA gene Swayne et al., 1997, 2000; Qiao et al., 2003). However, the available vaccines do not induce sterilizing immunity in chickens for a number of potential reasons, including lack of antigenic match between the vaccine and circulating strain and insufficient viral antigen in the vaccine. Agricultural influenza vaccines, unlike their human counterparts, are not standardized for antigen content and are delivered in adjuvants composed of water in oil emulsions (Stone, 1987). Such vaccines must be inexpensive because the profit margin in raising poultry is small. Reverse genetics was used to create an H5N3 vaccine for poultry (Liu et al., 2003) and appears to offer a means of preparing inexpensive, efficacious vaccines to highly pathogenic H5N1 influenza viruses. The N3 neuraminidase was used to allow distinction between infected and vaccinated animals (the DIVA strategy) (Capua et al., 2003). A dose of HA protein as small as 1.2 micrograms protected chickens from lethal H5N1 challenge and markedly reduced virus shedding by infected birds. Nevertheless, a small number of birds shed small amounts (depending on the vaccine dose) of H5N1 influenza virus (Liu et al., 2003).

There is limited information about the immunogenicity of influenza virus in ducks (Higgins et al., 1987). In recent studies, a reverse genetics-derived H5N1 vaccine containing the NA gene and modified HA gene of A/goose/Guangdong/1/96 (H5N1) and the other six genes from A/PR/8/34 (H1N1) was efficacious in chickens, geese, and ducks (Tian et al., 2005). That study firmly established the ability of reverse genetics derived, formalin inactivated, oil emulsion H5N1 vaccine to reduce disease signs and viral load in domestic poultry (including ducks and geese), with the possibility of controlling H5N1 at the source. While this study provided useful information, especially on vaccine efficacy in ducks and geese, each batch of vaccine contained different amounts of antigen, and there was no strategy for distinguishing between naturally infected and vaccinated birds. Additionally, as many as three doses of vaccine containing a very large quantity (13.8 μg) of HA were used in the goose study, and vaccine containing 4.6 μg of HA was used in the duck study.

In the present study we tested, in both chickens and ducks, the immunogenicity and efficacy of reverse genetics-derived vaccine containing standardized doses of H5 hemagglutinin derived from A/Chicken/Vietnam/C58/04 (H5N1) virus and the N3 neuraminidase of A/Duck/Germany/1215/73 (H2N3) virus. This vaccine was highly immunogenic and efficacious in both ducks and chickens and may therefore be useful for reducing the emergence of influenza viruses in both poultry and humans.

Results

Serologic responses of chickens to standardized H5N3 vaccine

The immune response of chickens to the standardized doses of inactivated H5N3 vaccine was determined by hemagglutination inhibition testing. Single doses of vaccine containing 0.25, 0.5, and 1.2 μg HA protein induced geometric mean HI titers of 254, 320, and 446, respectively (Table 1). After revaccination, these titers increased to 3171, 4101, and 4335, respectively. The placebo and unvaccinated control groups had no detectable HI antibody 21 days after initial vaccination, but one of the 25 placebo-vaccinated chickens had an HI titer of 1280 after revaccination. This unlikely occurrence did not result from introduction of live A/Chicken/Vietnam/C58/04 (H5N1) virus, or the bird would have died; the most likely explanation is the inadvertent administration of a dose of inactivated vaccine to this bird at the time of revaccination. Revaccination increased the HI antibody titers by about ten-fold, and all doses of vaccine induced high titers of HI antibodies.

Protective efficacy of standardized H5N3 vaccine in chickens

The efficacy of the H5N3 standardized inactivated vaccine in chickens was determined by comparing death and disease signs in the vaccinated, placebo-vaccinated, and unvaccinated

Table 1
Efficacy of inactivated H5N3 influenza vaccines in chickens

Vaccine dose	HI titer (GMT)			Tracheal shedding[a]					Cloacal shedding[a]					Post-challenge mortality
	Before vaccination	21 dpv	21 dpb	3 dpc	5 dpc	7 dpc	10 dpc	14 dpc	3 dpc	5 dpc	7 dpc	10 dpc	14 dpc	
1.2 μg	<10	446	4335	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25
0.5 μg	<10	320	4101	1/25	1/25[b]	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25
0.25 μg	<10	254	3171	1/25	1/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25
Placebo[c]	<10	<10	<10	1/1	Not tested—all birds dead				1/1	Not tested—all birds dead				24/24
No vac	<10	<10	<10	Not tested—all birds dead					Not tested—all birds dead					25/25

dpv = days post-vaccination; dpb = days post-boost; dpc = days post challenge; GMT = geometric mean titer.

[a] Number shedding/Number tested.

[b] Interpreted as a false positive—no corresponding HI or EID.

[c] One placebo-control bird was removed from the analysis because of error in revaccination.

groups (Table 1). Each of the three doses of vaccine provided complete protection from lethal challenge: none of the 75 vaccinated birds showed any clinical sign of infection. In contrast, all birds in the placebo group (minus the one aberrant bird) and all of the unvaccinated birds died by the fourth day post-challenge and showed disease signs typical of highly pathogenic avian H5N1 influenza. The vaccine efficacy was 100% (95% CI, 86%–100%).

To measure virus shedding by vaccinated and control groups, we obtained tracheal and cloacal swabs of all birds that were alive starting on the third day after challenge. One of the 25 birds that received the lowest dose of H5N3 vaccine (0.25 μg HA) had detectable tracheal virus on days 3 and 5 post-challenge (Table 1). Virus was detected only in undiluted samples and was not detected after day 5. One of the 25 birds receiving the intermediate dose (0.5 μg HA) shed detectable H5N1 virus on day 3 but not beyond, again at the lowest detectable level. In the group receiving the largest dose of vaccine (1.2 μg HA), none of the birds shed detectable virus. Thus, each dose of vaccine tested was highly efficacious in protecting chickens from infection.

Vaccination of ducks with standardized doses of inactivated H5N3 antigen

The available evidence indicates that free-range domestic ducks played an important role in the spread of H5N1 in Vietnam and Thailand (Gilbert and Slingenbergh, 2004). Backyard farms that raised both ducks and chickens were eight times as likely to be infected with H5N1 as farms that raised only chickens. Because no information was available about the immune response of ducks to standardized doses of influenza vaccine, we duplicated the chicken study in ducks. Two experiments were conducted, the first at the same antigen doses used in chickens (1.2, 0.5, 0.25 μg HA protein) and the second at lower antigen doses (0.25, 0.125, 0.0625, 0.0313 μg HA protein). Like the chickens, the ducks were vaccinated at 2 weeks of age, revaccinated at 5 weeks of age, and challenged 21 days after revaccination.

Serological and clinical effects of H5N1 vaccines in ducks receiving standardized doses of H5N3 vaccine in ducks

Although no death and disease signs were observed in ducks inoculated with A/Chicken/Vietnam/C58/04 (H5N1) virus, the H5N1 virus did replicate in ducks. In the first experiment in ducks, each of the doses of vaccine (1.2, 0.5, 0.25 μg HA) induced HI antibodies; geometric mean titers were 52, 45, and 72, respectively, 21 days after primary vaccination (Table 2). After revaccination, the titers rose to 220, 151, and 290, respectively. The placebo group had no detectable antibody after primary or secondary immunization. After oral and intratracheal inoculation with 30 50% chicken infectious doses (CID_{50}) of virus, all of the placebo-vaccinated group shed virus 3 days after challenge, whereas none of the group that received 1.2, 0.5, or 0.25 μg of HA protein shed detectable virus. One of the placebo-control ducks died between days 5 and 10 after challenge, while all 12 remaining ducks developed HI antibodies with titers of 40 to 320. The vaccine's efficacy in preventing virus isolation from tracheal swabs was 100% (95% CI, 76.43%–100%), 100% (95% CI, 73.06%–100%), and 100% (95% CI, 74.86%–100%) for vaccine doses of 0.25, 0.5, and 1.2, respectively. The vaccine's efficacy for preventing virus isolation from cloacal swabs was 100% (95% CI, 75.07%–100%), 100% (95% CI, 71.47%–100%), and 100% (95% CI, 73.39%–100%) for vaccine doses 0.25, 0.5, and 1.2, respectively.

In the second experiment, in which ducks received smaller doses of antigen (0.0313, 0.0625, and 0.125 μg HA), the HI antibody titers were <10, 21, and 15, respectively (Table 3). The lowest dose of vaccine (0.0313 μg HA) induced detectable HI antibody in only 5 of 10 ducks after primary vaccination. After vaccination with 0.0625 and 0.125 μg HA, eight of the 10 ducks in each group responded with detectable HI antibody. In the 0.25 μg HA group, all 10 ducks produced detectable antibody. After revaccination, all groups showed a marked increase in HI titers, which ranged from 92 (0.0313 μg) to 435 (0.25 μg).

After challenge of the vaccinated and control ducks with 30 CID_{50} of A/Chicken/Vietnam/C58/04 (H5N1) virus, only the unvaccinated control group showed replication of virus in 10/10 birds. No virus replication was detected in the ducks receiving 2 doses of 0.0313 or 0.0625 μg of HA antigen (Table 3). In contrast, 2 of 10 ducks in the 0.125 μg antigen group and 1 of 20 in the 0.25 μg antigen group shed virus for 1 day at the lowest detectable level. The vaccine's efficacy for prevention of tracheal shedding was 100% (95% CI, 68.05%–100%) after 2 doses of 0.0313 to 0.0625 μg HA and 94.44% (95% CI, 73.50%–99.82%) after two doses of 0.25 μg HA, in comparison

Table 2
Efficacy of inactivated H5N3 influenza vaccine in ducks at the doses used in chickens (first duck study)

Vaccine dose	Serology (HI GMT)			Tracheal shedding[a]				Cloacal shedding[a]				Post-challenge mortality
	21 dpv	21 dpb	25 dpc	3 dpc	5 dpc	10 dpc	17 dpc	3 dpc	5 dpc	10 dpc	17 dpc	
1.2 μg	52	220	209	0/13	0/13	0/13	0/13	0/13	0/13	0/13	0/13	0/13
0.5 μg	45	151	142	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
0.25 μg	72	290	177	0/14	0/14	0/14	0/14	0/14	0/14	0/14	0/14	0/14
Placebo	<10	<10	127	12/13	3/13	0/12	0/12	11/13	2/13	0/12	0/12	1/13

GMT = geometric mean titer. dpv = days post-vaccination. dpb = days post-boost. dpc = days post-challenge.
[a] Number shedding/Number tested.

Table 3
Efficacy of inactivated H5N3 influenza vaccine in ducks at lower doses of antigen (second duck study)

Vaccine dose	Serology (HI GMT)			Tracheal shedding [a]				Cloacal shedding [a]				Post-challenge mortality
	21 dpv	21 dpb	21 dpc	3 dpc	5 dpc	7 dpc	11 dpc	3 dpc	5 dpc	7 dpc	11 dpc	
0.25 μg [b]	37	435	253	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
0.25 μg [c]	35	367	367	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
0.125 μg	15	211	171	1/10	0/10	0/10	0/10	0/10	0/10	1/10	0/10	0/10
0.0625 μg	21	239	149	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
0.0313 μg	<10	92	121	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Placebo	<10	<10	345	9/10	3/9	0/9	0/9	9/10	2/9	1/9	0/9	1/10

MT = geometric mean titer. dpv = days post-vaccination. dpb = days post-boost. dpc = days post-challenge.
[a] Number shedding/Number tested.
[b] Batch 1.
[c] Batch 2.

to controls. The vaccine's efficacy for prevention of tracheal shedding after two doses of 0.125 μg HA was 88.89% (95% CI, 51.87%–99.65%) compared to control ducks commingled in the same pens.

Re-challenge of ducks with a highly lethal H5N1 strain

Because A/Chicken/Vietnam/C58/04 is non-pathogenic in ducks, we next tested whether this vaccine would protect ducks from challenge with an H5N1 strain highly lethal to ducks. We re-challenged the ducks that had been vaccinated with 0.25 μg, 0.5 μg, and 1.2 μg of A/Chicken/Vietnam/C58/04 (H5N1) and had been challenged once with A/chicken/Vietnam/C58/04 (H5N1) vaccine with A/Duck/Thailand/71.1/04 (H5N1) virus. This virus kills all ducks inoculated by the natural route of infection within 7 days and has an intravenous pathogenicity index (IVPI) of 2.87 in ducks (Sturm-Ramirez et al., 2005). Thus, these ducks were challenged a second time, first with the non lethal H5N1 strain then with the lethal H5N1 virus. The vaccinated ducks were divided into two groups; both groups were re-challenged with 10^7 EID_{50} of A/Duck/Thailand/71.1/04 (H5N1) virus, one group 30 days after the first challenge and one group 114 days after the first challenge.

None of the ducks showed disease signs and none shed detectable virus 3 and 5 days post-challenge (results not shown). Thus, the vaccine prepared from A/chicken/Vietnam/C58/04 (H5N1), a virus that is non-pathogenic in ducks and after challenge with the same viruses provides complete protection from re-challenge with a virus highly lethal to ducks. The protection after vaccination and initial challenge lasts for at least 114 days.

Single-dose vaccination of ducks

The above studies in chickens and ducks used a prime-and-boost regimen to ensure high antibody levels and efficacy. In practical terms, a single dose of vaccine would be preferable if it provided acceptable immunity. We therefore inoculated ducks with single doses of H5N1 vaccine, then drew blood for serology and challenged them 49 days later with the lethal A/duck/Thailand/71.1/04 (H5N1) strain. The doses of H5N3 vaccine ranged from 0.015 to 1.2 μg of HA. Prechallenge HI antibody titers ranged from <10 (0.015 μg HA) to 101 (0.5 μg HA) (Table 4). The HI titer of antibody to challenge virus did not increase after challenge in the vaccinated groups of ducks (Table 4) but did increase in the placebo-control group. This finding indicated

Table 4
Efficacy of a single dose of inactivated H5N3 influenza vaccine in ducks after challenge with a duck-lethal H5N1 strain [a] (third duck study)

Vaccine dose	Serology (GMT)		Tracheal shedding [b]			Cloacal shedding [b]			Post-challenge mortality
	Pre-chall	Post-chall	4 dpc	7 dpc	11 dpc	4 dpc	7 dpc	11 dpc	
1.2 μg	40	20	0/8	0/8	0/8	0/8	0/8	0/8	0/8
0.5 μg	101	180	0/6	0/6	0/6	0/6	0/6	0/6	0/6
0.25 μg	40	32	0/7	0/7	0/7	0/7	0/7	0/7	0/7
0.125 μg	15	13	0/7	0/7	0/7	0/7	0/7	0/7	0/7
0.0625 μg	14	11	0/8	0/8	0/8	0/8	0/8	0/8	0/8
0.0313 μg	<10	<10	0/8	0/8	0/8	0/8	0/8	0/8	0/8
0.015 μg	<10	<10	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Placebo	<10	135	12/12	2/6	0/6	8/12	0/6	0/6	8/12 [c]

Serology values indicate HI titers; reisolation values indicate number positive/total.
[a] Ducks were challenged with the duck lethal A/duck/Thailand/71.1/04 (H5N1) strain.
[b] Number shedding/Number tested.
[c] Two ducks with severe clinical signs euthanized on day 11.

Table 5
Pilot study of single vs. double vaccination of khaki-Campbell ducks[a]

Treatment	Serology (HI GMT)			Tracheal shedding[b]			Cloacal shedding[b]			Post-challenge mortality
	11 dpv	11 dpb	14 dpc	2 dpc	4 dpc	7 dpc	2 dpc	4 dpc	7 dpc	
One vaccination	35	85	368	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Two vaccinations	25	640	1280	0/10	0/10	0/10	0/10	0/10	0/9[c]	0/9
Unvac. control	<10	<10	1280[d]	10/10	5/5	0/3	9/10	3/5	0/3	8/10
Contact control	<10	<10	<10	n.d.	0/10	0/10	n.d.	0/10	0/10	0/10

[a] Ducks were challenged with the duck lethal A/duck/Thailand/71.1/04 (H5N1) strain.
[b] Number shedding/Number tested.
[c] One duck euthanized with no clinical signs and no virus isolation.
[d] Titers from two control ducks that survived challenge.

that the challenge virus did not replicate in the vaccinated groups.

No mortality or disease signs were detected in any of the vaccinated ducks. In contrast, 8/12 of the placebo group died and the remaining had severe disease signs including neurological abnormality and/or cloudy eyes. Vaccine efficacy for prevention of mortality in pens of ducks vaccinated with the 1.2, 0.0625, 0.0313 or 0.015 μg doses of vaccine was 100% (95% CI, 34.94%–100%). The vaccine efficacy for the prevention of mortality in pens of ducks administered the 0.125 and 0.25 μg doses of vaccine was 100% (95% CI, 38.63%–100%) as compared to controls. The vaccine efficacy for the prevention of mortality in pens of ducks administered the 0.5 μg dose of vaccine was 100% (95% CI, 22.03%–100%) compared to controls.

It is noteworthy that despite the absence of detectable HI antibody in the prechallenge sera of ducks vaccinated with 0.015 μg and 0.0313 μg of HA, there was no detectable virus replication after challenge and no disease signs or deaths, whereas virus replication was detectable in the tracheas of 12/12 ducks that received placebo, and 8/12 died. Infectivity neutralization tests of duck sera that showed no HI activity failed to detect neutralization of the homologous virus (data not shown).

Pilot vaccination study of Khaki Campbell ducks

Because many of the ducks raised in Thailand for egg production and as backyard poultry are Khaki Campbell ducks, a pilot study was done to determine the efficacy of inactivated oil emulsion H5N3 vaccine in this species. A dose of vaccine containing 0.25 μg of HA antigen was administered to groups of 10 ducks; one group received a single dose of vaccine and the other received a boost dose after 2 weeks. The ducks were inoculated intratracheally and intranasally with 10^4 EID_{50} (180 DLD_{50}) of the lethal A/duck/Thailand/71.1/04 (H5N1) strain 14 days after vaccination. After either one or two doses of vaccine, Khaki Campbell ducks were completely protected from challenge with the lethal A/duck/Thailand/71.1/04 (H5N1) strain (Table 5). All of the unvaccinated control ducks showed severe neurological symptoms and cloudy eyes, 8/10 died, and the remaining two were severely affected. All 10 control birds shed virus from both the trachea and cloaca by day 2. In contrast, no virus was detected in the vaccinated birds on any day, and the birds remained completely healthy. At day 3 after challenge of the vaccinated birds, unvaccinated ducks were placed in their cages to test possible virus transmission. None of the 5 control birds showed disease signs and none shed detectable virus or had antibody to the challenge virus. The HI antibody titer induced by the vaccine ranged from 35 after a single dose of vaccine to 640 after a boost dose.

Demonstration of DIVA

One of the concerns about the use of influenza vaccines in poultry is the ability to distinguish between naturally infected and vaccinated birds. This can be achieved by using an irrelevant neuraminidase antigen in the vaccine—a concept introduced by Capua et al. (2003), called "In distinguishing infected and vaccinated animals"—DIVA. In Table 6, we demonstrate that before challenge unvaccinated ducks had no detectable antibodies to H5 in HI assays and background levels

Table 6
Neuraminidase inhibition titer: comparison with hemagglutination inhibition serum antibody titer

Number of doses	Dose (μg)	Animal	Hemagglutination inhibition titer		N1 neuramindase inhibition titer		N3 neuraminidase inhibition titer	
			Pre-challenge	Post-challenge	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
One	0	Duck-2	<	40	<	<	20	20
	0.5	Duck-13	640	1280	<	<	>160	>160
		Duck-67	160	160	<	<	>160	>160
Two	0.25	Duck-436	2560	1280	<	<	>160	>160
		Chick-32	5120	5120	<	<	>160	>160
		Chick-53	10,240	5120	<	<	>160	>160
	<, less than 1:10							

of antibodies to N3 in NI assays (1:20). After H5N1 infection the unvaccinated ducks demonstrated a small rise in level of antibodies to H5 (1:40), but no antibodies to N1. In contrast, birds vaccinated with H5N3 had high levels of antibodies to H5 and N3 but no detectable antibodies to N1 before or after challenge with H5N1 virus.

Discussion

Domestic ducks and chickens appear to be the immediate source of the H5N1 viruses that have infected humans recently in southeast Asia, and ducks (particularly free-range ducks) are associated with H5N1 infection in domestic chickens in Thailand (Gilbert and Slingenbergh, 2004). After outbreaks are initially controlled by culling infected flocks, vaccination can help to provide continued control. Here, we have reported the preparation and testing of a reverse genetics-derived H5N3 vaccine that induces protective immunity to highly pathogenic H5N1 viruses in chickens and ducks. Doses of 0.25, 0.5, and 1.2 μg of A/Chicken/Vietnam/C58/04 (H5) HA protein completely prevented disease signs in chickens, whereas the placebo-control and unvaccinated chickens died. Only two of 50 vaccinated chickens shed virus from the cloaca at the lowest level of detection, with infectivity titers less than 1.0 $\log_{10}$/ml.

The high titers of antibody induced in chickens after a single 0.25, 0.5, or 1.2 μg dose of vaccine suggest that a single immunization with these doses of antigen would provide protective immunity. Additional studies are planned to test this hypothesis. The minimal dose of antigen required to provide protective immunity was not determined in these studies, nor whether doses below 0.25 μg HA protein are efficacious.

In ducks, either single or double doses of inactivated H5N3 oil emulsion vaccine induced protective immunity against challenge with either less pathogenic or highly pathogenic strains of H5N1 influenza virus. Antigen doses ranging from 0.25 to 1.2 μg of HA protein induced HI antibody titers greater than 40; doses of 0.0625 to 0.125 μg induced detectable HI titers of 15, while doses of 0.015 to 0.0313 μg did not induce detectable HI antibody. However, all ducks vaccinated with the lower antigen doses were protected from challenge with lethal H5N1 virus. In the four different studies we conducted in ducks, only three vaccinated birds shed virus after challenge, and then only at the lowest detectable level.

Protection of vaccinated ducks from challenge with H5N1 virus in the absence of detectable HI antibody suggests that either the sensitivity of the test is low or the vaccine induces cellular immune responses. The duck sera that showed no HI activity after vaccination failed to neutralize homologous virus (data not shown). Thus, protection in the absence of detectable antibody remains unexplained; it may have a cell mediated basis or may be due to unknown properties of duck immunoglobulins (Higgins et al., 1987).

The complete protection of ducks by a single low dose of HA antigen (0.015 μg or more) suggests the feasibility of using influenza vaccine in the field to control the spread of H5N1 virus in ducks. The low effective dose indicates that multiple doses of vaccine can be produced per embryonated chicken egg and that unconcentrated allantoic fluid containing virus could be diluted to produce multiple doses of vaccine. Tian et al. (2005) provided the only other available information about the efficacy of H5N1 influenza vaccines in ducks; they reported that vaccine containing 4.6 μg of HA was efficacious in ducks. Our findings indicate that much lower doses of vaccine are efficacious in ducks; this disparity may reflect differences between the adjuvant preparations used.

Our findings provide preliminary information about the duration of protection provided by vaccination of ducks; rechallenge after 114 days with the highly lethal duck strain of H5N1 induced no disease signs and no virus shedding. However it must be kept in mind that these ducks had been challenged once with the chicken H5N1 virus and were therefore immunologically boosted before the rechallenge 114 days later. A single dose of vaccine may be sufficient for meat ducks, while a booster dose may be needed to provide longer immunity for laying ducks.

The widespread raising of free-range ducks in Vietnam and Thailand after the rice harvest may have contributed to the initial spread of H5N1 in 2003–2004; such ducks can be transported by truck over relatively long distances. The rapid evolution of the H5N1 virus in ducks between 2002 and 2005 and the emergence of variants that do not kill ducks but remain lethal to chickens (Sturm-Ramirez et al., 2005; Hulse-Post et al., 2005)are factors that contribute to the continuing circulation of H5N1 viruses in southeast Asia. Not only are free-range ducks raised, but the presence of ducks in most backyards in the villages and towns of Asia are a continuing challenge to the control of H5N1 influenza, especially when the circulating strains are nonpathogenic in ducks. In 2005, Vietnam banned the hatching of duck eggs in an attempt to reduce the risk of H5N1 infection in poultry and humans. Our results add support to the findings of Tian et al. (2005) that the use of oil emulsion H5 vaccines in ducks offers an option for controlling H5N1 virus at the source.

Ducks are immunized by low doses of H5 antigen in oil emulsion adjuvant, whereas high doses of the human counterpart of this virus (A/Vietnam/1203/04 [H5N1]) are required to immunize ferrets and humans. In ferrets, 7 μg of antigen induced protective immunity (Hoffmann et al., 2005), but adjuvants were not used.

The apparent spread of H5N1 viruses from domestic ducks to wild migratory birds in Qinghai Lake, China (Chen et al., 2005) raises the question whether widespread use of vaccine contributed to or could have prevented this transfer of H5N1 virus. There is continuing controversy about the use of vaccines in poultry and whether they contribute to or reduce antigenic drift in this virus. Replication of H5 avian influenza virus was demonstrated in vaccinated chickens, with the possibility of transmission to unvaccinated birds (Swayne et al., 2001). On the other hand, studies on poultry farms in Hong Kong demonstrated that H5 vaccine could interrupt transmission of an ongoing outbreak of H5N1 avian influenza (Ellis et al., 2004). The absence of H5N1 influenza virus infection in humans and domestic poultry in Hong Kong in 2004–2005 after adoption of vaccination with inactivated H5 vaccine, together with

improved sanitation, attests to the potential for efficacious field use of an H5 vaccine.

An important concern about poultry influenza vaccines is the use of substandard or unstandardized vaccines. Some lots of H5N2 vaccines from Mexico protected against disease signs but did not prevent virus shedding and transmission to unvaccinated birds (Garcia et al., 1998). This effect may be a factor in the continued presence and antigenic drift of H5N2 virus in Central America (Lee et al., 2004). While veterinary influenza vaccines with different compositions of oil emulsion adjuvants may differ in their effectiveness, a case could be made for considering the creation of international standards for antigen content of veterinary influenza vaccines, as is the case for human influenza vaccines. Veterinary vaccines should contain, for example, no less than the minimal dose of antigen (in terms of μg HA protein) demonstrated to have a pre-determined standard of effectiveness. Cooperation between companies selling vaccine internationally and international organizations such as FAO and OIE in the development of such standards would be likely to have significant value.

Because the highly pathogenic H5N1 avian influenza virus is endemic in domestic poultry in Eurasia and may be established globally in wild migratory birds, there will be a need for high-quality standardized vaccine. The current report describes a reverse genetics-derived, inactivated, oil emulsion vaccine that is effective in both chickens and ducks and can be produced without virus concentration or purification. The possibility still exists that the use of such a vaccine could prevent transmission of H5N1 influenza virus to humans and prevent the emergence of a pandemic strain. There is an urgent need for additional regulation of veterinary influenza vaccines because of concern that substandard and inadequately inactivated vaccines may have contributed to outbreaks of H5N1 and H5N2 disease in Asia in 2005.

Materials and methods

Viruses

A/Chicken/Vietnam/C58/04 (H5N1) virus was isolated from a moribund infected chicken and grown in chicken embryos. This virus meets the definition of a highly pathogenic avian influenza virus, possessing a series of basic amino acids at the cleavage site of the HA and killing experimentally infected chickens in less than 1 day. The intravenous pathogenicity index (IVPI) in chickens is 3.0. A/Duck/Germany/1215/73 (H2N3) virus was obtained from the influenza virus repository at St. Jude Children's Research Hospital. The A/PR/8/34 (H1N1) virus (PR8) is the strain used in the preparation of human influenza vaccines. The highly pathogenic H5N1 viruses were handled in BSL3+ facilities approved for studies with these viruses.

Reverse genetics

RT-PCR was performed with segment-specific primers as previously described (Hoffmann et al., 2000). Briefly, RNA was isolated by using the RNeasy kit (Qiagen) and transcribed to cDNA with the Uni12-primer (AGC AAA AGC AGG). Plasmids encoding the PB1, PB2, PA, NP, M, and NS genes of influenza PR8 were constructed as previously described (Hoffmann et al., 2002) and were designated pHW191-PB2, pHW192-PB1, pHW193-PA, pHW195-NP, pHW197-M, and pHW198-NS. The plasmid, which encoded the HA of the A/Chicken/Vietnam/C58/04 (H5N1) virus, with a deletion of the polybasic amino acid region at the HA1–HA2 cleavage site, was derived by PCR amplification of two fragments.

The fragments were digested with *Bsm*BI and inserted into pHW2000-*Bsm*BI by a three-fragment ligation reaction. The N3 NA of influenza A/Duck/Germany/1215/73 was cloned into the pHW2000 vector by using PCR and the N3-specific primers previously described (Liu et al., 2003). Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital determined DNA template sequence using Big Dye Terminator(v.3) chemistry and synthetic oligonucleotides. Samples were analyzed on Applied Biosystem 3700 DNA Analyzers.

Generation of recombinant viruses

Recombinant viruses were generated by DNA transfection as described by Hoffmann et al. (2002) and Liu et al. (2003).

Preparation of vaccines

Viruses were propagated in the allantoic cavities of 10- to 11-day-old embryonated chicken eggs at 35 °C for 48 h. Allantoic fluid was harvested, and virus was inactivated by adding 0.2% formalin and allowing the fluid to remain at 37 °C for 24 h. Inactivation was confirmed by the absence of detectable infectivity after two blind passages of the treated allantoic fluid in embryonated eggs.

The content of the hemagglutinin protein in the allantoic fluid was standardized by the single radial immunodiffusion technique as described previously (Wood et al., 1985), using monospecific antiserum to the HA of A/Vietnam/1203/04 (H5N1) (Wood et al., 2001).

Vaccines were prepared from inactivated, unconcentrated allantoic fluid of reverse genetics A/Chicken/Vietnam C58/04 (H5N1) standardized for hemagglutinin (HA) protein concentration. Placebo vaccines contained virus-free allantoic fluid from 12-day-old embryonated chicken eggs. The vaccines were formulated in standard water in mineral oil emulsion.

Test animals

Chickens

SPF white leghorn chickens (*Gallus domesticus*) from Charles River were wing-banded and provided feed and water ad libitum. The birds were housed in pens in BSL3+ facilities; groups of 25 birds received their first dose of vaccine at 2 weeks of age, were revaccinated at 5 weeks of age, and were challenged with 30 chicken lethal doses of A/Chicken/Vietnam/C58/04 (H5N1) at 8 weeks of age. Housing in pens was such

that an equal number of chickens from each treatment group were housed in each pen.

Ducks

Outbred Pekin white ducks (*Anas platyrhynchos*) from Ideal Poultry, Cameron, TX, were leg-banded and housed as described above for the chickens with the exception that due to constraints in availability of the BSL3+ facilities ducks could not be arranged with an equal number from each treatment group housed in each pen.

Animal studies

Vaccination and blood sampling

Chickens were randomly assigned to groups and vaccinated intramuscularly in the breast. Ducks were randomly assigned to groups and were vaccinated intramuscularly in the legs (one half dose in each leg). Primary vaccination was given at 2 weeks of age and revaccination at 5 weeks of age. Blood samples were obtained from all birds before each vaccination and before challenge. The final blood sample was obtained 74 days after primary vaccination.

Challenge and swab sampling

Three weeks after revaccination, chickens and ducks were challenged by intranasal instillation of 1.0 ml containing 30 CLD_{50} of A/Chicken/Vietnam/C58/04 (H5N1) virus. All birds were observed daily for mortality. From day 3 through day 17 post-challenge, tracheal and cloacal swabs were collected on designated days (2, 3, 4, 5, 7, 10, 11, 14, 17 depending on the experiment) from all living birds for virus isolation in chicken embryos.

In the single-vaccination duck experiment, the birds were challenged by intranasal instillation of 1.0 ml containing 100 duck lethal doses of A/duck/Thailand/71.1/04 (H5N1) virus. The birds were observed daily for mortality. Four, 7, and 11 days after challenge, tracheal and cloacal swabs were collected from all living birds for virus isolation.

Serologic tests

Hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests were done as described previously (Webster et al., 2002).

Statistical analysis

The vaccine efficacy statistics and 95% exact confidence intervals were calculated based on the bird using the inverted two one-sided tests methods in PROC-STATXACT (Cytel Software Corp., Cambridge, MA).

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